

Spatial and Temporal Expression of Glucocorticoid, Retinoid, and Thyroid Hormone Receptors Is Not Altered in Lungs of Congenital Diaphragmatic Hernia

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ABSTRACT: The degree of associated pulmonary hypoplasia and persistent pulmonary hypertension are major determination factors for survival in congenital diaphragmatic hernia (CDH) patients. Glucocorticoids, thyroid hormone, and vitamin A have been shown to be involved in human lung development. To determine their therapeutic potential in hypoplastic lungs of CDH patients, the temporal and spatial expression of glucocorticoid receptor, thyroid hormone receptors, retinoic acid receptors, and retinoid X receptors were evaluated in lungs of CDH patients, hypoplastic lungs from other causes, and normal lungs. As a series of supportive experiments, the expressions of these receptors were analyzed in lungs of nitrofen-induced CDH rats. Immunohistochemistry (human and rat) and *in situ* hybridization (rat) demonstrated no overt difference between CDH, hypoplastic, and control lungs, either in the localization nor the timing of the first expression of all analyzed receptors. The mRNA expression of each receptor was detected in all human CDH lungs by quantitative PCR. Our results suggest that, as far as receptors are concerned, hypoplastic lungs of fetuses and newborns with CDH are potentially as responsive to glucocorticoids, thyroid hormone, and retinoic acid as the lungs of normal children. (*Pediatr Res* 60: 693–698, 2006)

CDH occurs approximately 1 in 2500 live births. This malformation, first described in 1848 by Vincent Bochdalek, is characterized by a diaphragmatic defect, herniation of abdominal viscera into the thoracic cavity, a variable extent of PH, and PPH. Despite the recent progress in new therapeutic strategies such as inhaled nitric oxide, exogenous surfactant therapy, or extracorporeal membrane oxygenation in selected infants, newborns with CDH continue to experience a high mortality (1,2). The major factors limiting survival of CDH patients are respiratory insufficiency secondary to PH and PPH. The lungs of newborns with CDH are characterized by a variable amount of immature alveoli with thickened walls

and increased interstitial tissue, whereas surfactant deficiency may occur mainly due to surfactant inactivation instead of a primary surfactant deficiency (3).

With the increased use of prenatal ultrasound, more CDH cases are being diagnosed prenatally. Failure of postnatal therapies to significantly improve the prognosis of these infants has led to new strategies to rescue or stimulate fetal lung growth before delivery. For over two decades, pioneer efforts have been made to repair the diaphragmatic defect prenatally in fetuses with CDH but the outcome has been hampered by technical difficulties and a high rate of preterm delivery (4). The observation that infants with laryngeal atresia develop hyperplastic lungs led to the concept of “PLUG” (plug the lung until it grows) and fetal endoscopic temporary tracheal occlusion (FETENDO) (5,6). A randomized clinical trial sponsored by the National Institutes of Health demonstrated no survival benefit compared with elective delivery in the specialist centers with optimal postnatal care (4). However, the trial continues in Europe with fetoscopic tracheal occlusion (FETO), hoping that further technical refinement may improve survival in high-risk patients (7,8).

Inasmuch as the benefit of fetal surgical interventions remains unclear, pharmacological approaches to stimulate prenatal lung growth and maturation have been considered as another strategy to improve lung development in case of CDH. GC are used worldwide to accelerate pulmonary development in threatened premature labor to decrease the incidence of respiratory distress syndrome (9,10). Within this context, the potential beneficial effects of prenatal GC treatment on lung maturation have been studied in the nitrofen (2, 4-dichlorophenyl-p-nitrophenyl ether)-induced CDH rat model and the surgical sheep model for CDH. The nitrofen model, based on the teratogenic effects of the herbicide, lead to a condition in

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Abbreviations: CDH, congenital diaphragmatic hernia; GC, glucocorticoids; GR, glucocorticoid receptor; PH, pulmonary hypoplasia; PPH, persistent pulmonary hypertension; RA, retinoic acid; RAR, retinoic acid receptors; RXR, retinoid X receptors; TR, thyroid hormone receptor

rodents very similar to that observed in human CDH, including the features of PH and PPH (11). A disturbance in maternal thyroid function as well as in fetal thyroid function has been suggested to be involved in the teratogenic effect of nitrofen. Firstly, there is a stereo-chemical similarity between nitrofen and thyroid hormone. Secondly, nitrofen-treated dams have less malformed fetuses if T₄ is given during pregnancy; and, thirdly, nitrofen-treated pregnant rats have lowered T₄ and TSH levels (12). In addition, our group has demonstrated that nitrofen decreases the binding of T₃ to the thyroid hormone receptor α 1 and β 1 in a noncompetitive way (13).

Recently, more attention has been drawn to vitamin A and its active metabolite, RA. The classical data published by Anderson (14) showed that 25% of rats born from dams with a vitamin A-deficient diet have a defect in the diaphragm. The incidence of a diaphragmatic defect reduced from 31% to 24% when a single dose of vitamin A administered to vitamin A-deficient female rats on d 15 of pregnancy (15). Thebaud *et al.* (16,17) have shown that antenatal treatment with vitamin A in the nitrofen-induced CDH model reduces the incidence of CDH at term as well as improving lung growth and maturation. In humans, lower plasma vitamin A levels were found in prematurely born infants, especially in patients with respiratory distress and in a selective number of term newborns with pulmonary hypoplasia associated with congenital diaphragmatic hernia (18,19). Although exciting, the confirmation of these findings awaits international collaboration, which is ongoing at present.

The actions of glucocorticoids, thyroid hormone, and retinoic acid are mediated through their specific nuclear receptors and the level of nuclear receptor expression determines cellular sensitivity to certain hormones (20). Since the optimal way of prenatal modulation of CDH is still a matter of ongoing debate, detailed information on the tissue distribution of these receptors in case of abnormal pulmonary development is warranted. Therefore, we examined the expression of GR- α , TR, RAR, and RXR in human lungs with CDH, pulmonary hypoplasia due to other causes and compared these data with an ontogenic study of the same receptors during normal human lung development published by our group (21). As a series of supportive experiments, we examined the expression of these receptors in the lungs of nitrofen-induced CDH rats.

MATERIALS AND METHODS

Human. Following approval of the experimental design and protocols by the University Ethical Committee, lung tissues were retrieved from the archives of the Department of Pathology, Erasmus MC, Rotterdam. There were 18 CDH lung samples collected from either termination of pregnancy or patients who died within 48 h after birth (gestational age, 18–41 wk; mean, 34.2 wk). Demographic information of CDH cases is shown in Table 1. Immunohistochemical studies were performed in all CDH samples, whereas PCR was performed in five samples (cases 1–5) of which frozen tissue was available. Twenty paraffin-embedded lung samples from fetuses or newborns with pulmonary hypoplasia due to other causes, including Pena-Shokeir syndrome, hydrothorax, renal dysgenesis, and oligohydramnios (gestational age, 18–40 wk; mean, 30.5 wk) were included for immunohistochemical studies. None of the patients included in this study was subjected to prenatal steroid or extracorporeal membrane oxygenation therapy. Lung tissue from 15 age-matched fetuses and newborns (from termination of pregnancy or autopsies) without pulmonary abnormalities served as control (gestational age,

Table 1. Clinical information of congenital diaphragmatic hernia patients

Case	Gestational age (wk)	Birth weight (g)	Lung/body weight ratio*	Time of death after birth
1†	23	558	0.012	Termination of pregnancy
2†	23.5	350	0.013	Termination of pregnancy
3†	34.5	2800	0.008	<24 h
4†	38	2450	0.011	Minutes
5†	41	3510	0.006	48 h
6	18	161	0.012	Termination of pregnancy
7	21	416	0.007	Termination of pregnancy
8	28	830	0.003	2 h
9	34	1250	0.005	Minutes
10	36	2515	0.003	1 h
11	38	2870	0.005	30 min
12	38	2900	NA	24 h
13	39	2890	0.003	15 min
14	40	3225	<0.01	6 h
15	40	1220	NA	24 h
16	40	2050	0.005	Minutes
17	41	2765	0.005	1.5 h
18	42	3355	0.002	30 min

* Normal lung/body weight ratio: before 25 wk of gestation = 0.015; from 25 wk of gestation = 0.012.

† Frozen lung tissues available for quantitative PCR.

18–41 wk; mean, 30.4 wk). All samples were harvested within 24 h after death.

Nitrofen-induced rats. Adult Wistar rats were obtained from the HSD animal farm in Zeist, the Netherlands. Animal experiments were performed in accordance with the guidelines of the animal research committee. The positive sperm plug day was designated as d 1 of gestation. To induce CDH and pulmonary hypoplasia, 100 mg of nitrofen dissolved in 1 mL olive oil was given to the dams by gavages on d 10 (11). Fetuses were delivered by cesarean section at 15, 18, and 20 d of gestation, which corresponds to the pseudoglandular, the canalicular, and the sacular stage of rat pulmonary development respectively (term = 23 d). All tissues were fixed in 4% buffered formaldehyde and embedded in Paraplast Plus (Monoject, Kildare, Ireland). Seven-micrometer sections were cut and mounted onto RNase free 3-aminopropyltriethoxysilane coated slides (Sigma Chemical Co., St. Louis, MO). There were three to five random fetuses in each experimental group. From the group treated with nitrofen, only fetuses with a diaphragmatic hernia were included in this study.

Immunohistochemistry. Immunohistochemistry was performed on human and rat tissues for GR α , TR α , TR β , RAR (α, β, γ), and RXR (α, β, γ) using a ChemMate DAKO EnVision Detection Kit, Peroxidase/DAB, Rabbit/Mouse (DakoCytomation B.V., Heverlee, Belgium). Primary antibodies and immunohistochemical processes were described in detail in our previous study (21). Negative controls were performed by omission of the primary antibodies. No quantification of the staining intensity was attempted because the immunohistochemical study was basically to demonstrate the cellular distribution of the receptors.

In Situ hybridization. Rat tissues were analyzed for GR, TR α , TR β , RXR α , and RXR β . GR cDNA (2.4 kb) was isolated from rat liver and recloned in pBluescript SK+ in the *Bam*HI restriction site of the multiple cloning site (kind gift from Dr. Paul Godowski) (22). Probes for the TR α and TR β mRNA in *Eco*RI-*Hind*III cDNA fragments were obtained from H.C. Towle (23). RXR α and RXR β mRNA were detected using an *Eco*RI-*Eco*RI cDNA fragment (1850 bp) and *Eco*RI-*Hind*III cDNA fragment (1360 bp), respectively. The hybridization conditions were as described elsewhere (24). The sections of rat fetuses were digested with 0.1% (wt/vol) pepsin (Sigma Chemical Co.) in 0.01 M HCl for 7 min (d 15 of gestation), 10 min (d 18 of gestation), and 15 min (d 20 of gestation) at 37°C. The hybridization with [α -³⁵S] dUTP labeled anti-sense probes was 16–18 h at 54°C. The probe concentration was approximately 50 μ g/ μ L and the specific activity of the anti-sense RNA probe was approximately 500 dpm/pg. After hybridization, the sections were washed and treated with RNase A. After exposure to photographic emulsion (Ilford Nuclear Research Emulsion G-5, Ilford,

Cheshire, UK), the sections were counterstained with nuclear fast red, dehydrated in a graded series of ethanol and xylol, and mounted in Malinol (Chroma-Gesellschaft, Schmidt GmbH+Co, Köngen, Germany).

Real-time quantitative PCR. Five CDH lungs and 15 control human lungs were analyzed for mRNA expression of GR α , TR α 1, TR β , RAR (α,β,γ), and RXR (α,β,γ). Total RNA was extracted from frozen human lung tissues using TRIzol reagent (Invitrogen, Breda, the Netherlands), according to the manufacturer's instruction. Total RNA was quantified by measuring the absorbance at 260 nm and the purity was checked with 260/280 nm absorbance ratio. Reverse transcription and PCR conditions were carried out exactly as described before (21). PCR was run in triplicate for each sample. Negative control samples and reactions mixed without cDNA templates were run in parallel.

PCR results are shown as the relative expression level ($2^{-\Delta\Delta Ct}$) of normalized samples (ΔCt) in relation to the expression of the "calibrator" sample. The Ct value refers to the cycle number at which the PCR plot crosses the threshold line. ΔCt is calculated by subtracting Ct value of the corresponding GAPDH control (endogenous reference control) from the specific Ct value of the target, and $\Delta\Delta Ct$ is obtained by subtracting the ΔCt of each experimental sample from the ΔCt of the "calibrator" sample. In this study, the control lung of 18 wk of gestation were used as a calibrator, which was arbitrarily set at 100% (arbitrary value = 1).

RESULTS

Human. Five CDH lungs and 15 age-matched controls were analyzed with quantitative PCR. The mRNA expression for GR α , TR α 1, TR β , RAR (α,β,γ), and RXR (α,β,γ) was detected in all samples and the relative mRNA expression level of each gene is illustrated in Figure 1. No statistic analysis was done due to limited number of CDH samples. The lungs of 18 CDH cases and 20 patients with pulmonary hypoplasia secondary to causes other than CDH were included for immunohistochemical studies. The expression of GR α , TR α , TR β , RAR (α,β,γ), and RXR (α,β,γ) was detected in a similar fashion in all control, CDH, and pulmonary hypoplasia samples. GR α reactivity was detected mainly in epithelial cells (results not shown). TR α was expressed in both mesenchymal and epithelial cells (Fig. 2, A–C), whereas TR β reac-

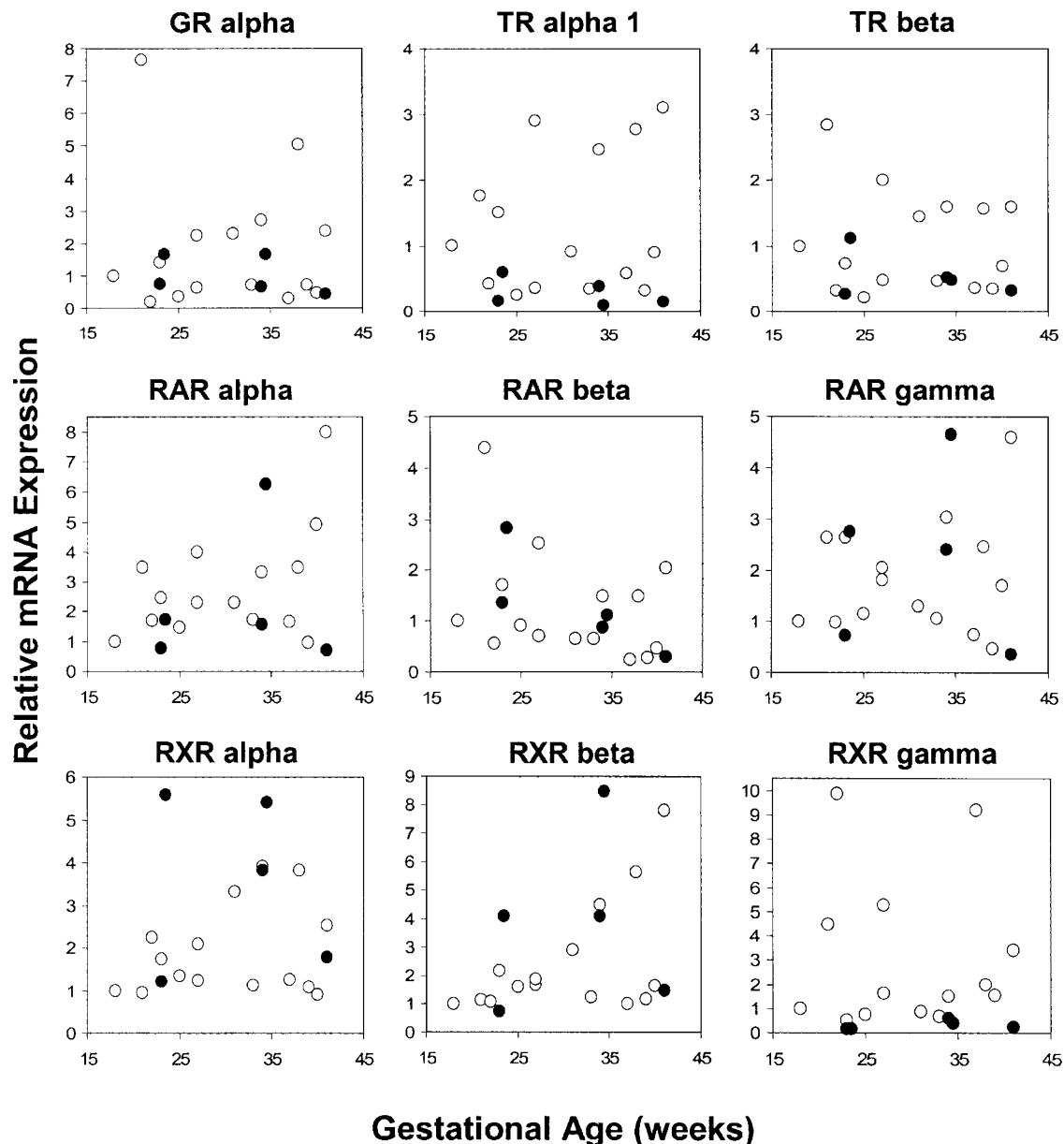


Figure 1. Relative mRNA expression of GR α , TR α 1, TR β , RAR (α,β,γ), and RXR (α,β,γ) in human CDH and control lungs. All analyzed receptors were expressed in five lungs from CDH cases (●) and 15 control lungs (○). The expression levels are shown as relative value in an arbitrary unit compare with control lung of 18 wk of gestation (as outlined in "Materials and Methods").

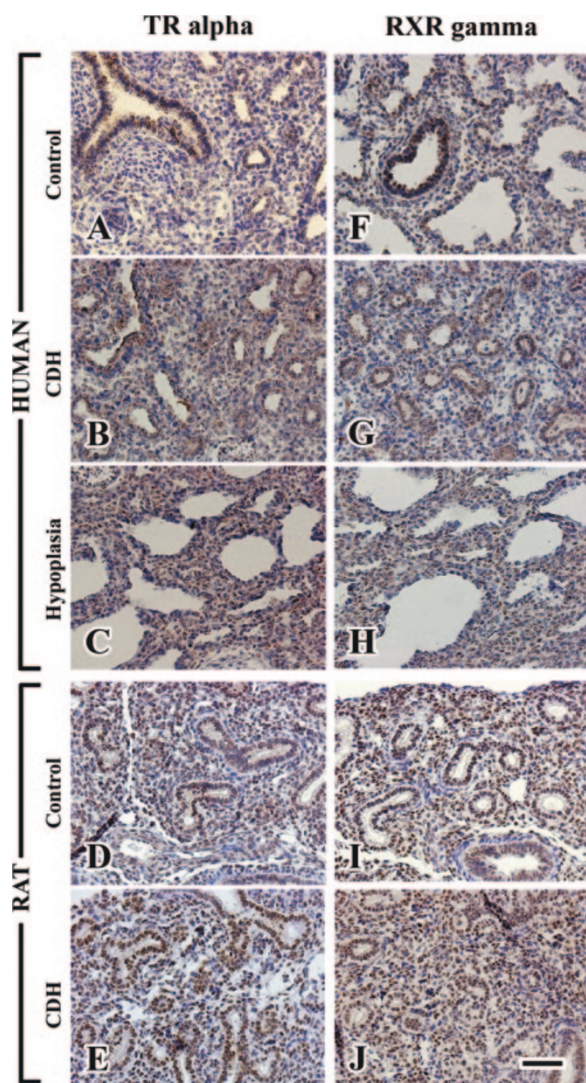


Figure 2. TR α and RXR γ expression pattern in human and rat lungs. TR α (A–E) and RXR γ (F–J) are seen in both epithelial and mesenchymal cells. There is no difference in the localization of TR α or RXR γ in the lungs of human control; 23 wk (A, F), CDH; 34 wk (B, G), or pulmonary hypoplasia due to other causes; 27 wk (C, H). Comparable patterns are observed in control (D, I) and nitrofen-induced CDH (E, J) rat lungs (d 18 of gestation). All pictures were taken at the same magnification; scale bar = 100 μ m.

tivity was detected in the epithelial cells as well as in the endothelial cells of arteries (results not shown). RAR (α, β, γ) and RXR (α, β, γ) were expressed in virtually all epithelial and mesenchymal cells (Fig. 2, F–H, for RXR γ , for others results not shown). The spatio-temporal expression patterns of all receptors were similar in the CDH lungs, pulmonary hypoplasia due to other causes, and normal lungs.

Nitrofen-induced rat. In a series of supportive experiments, the immunohistochemical studies demonstrated the expression of GR α , TR α , TR β , RAR (α, β, γ), and RXR (α, β, γ) at the protein level in both control and nitrofen-induced CDH rat lungs. The staining pattern of each receptor was comparable to that observed in human lungs as shown in Figure 2 for TR α (Fig. 2, D and E) and RXR γ (Fig. 2, I and J), for others the results are data not shown. The results of the RNA *in situ* hybridization studies at d 15 and d 18 of gestation are shown in Figures 3 and 4, respectively. GR mRNA was observed in

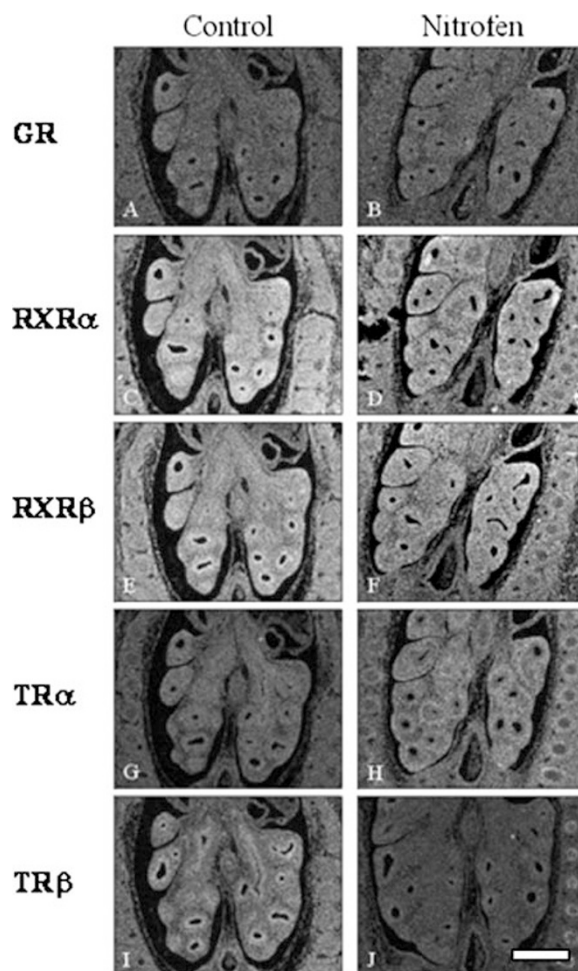


Figure 3. *In situ* hybridization studies of hormone receptors expression in normal (A, C, E, G) and nitrofen-induced CDH rat lungs (B, D, F, H) at d 15 of gestation. GR mRNA is observed in the endoderm lining the branching airways (A, B). RXR α mRNA (C, D) and RXR β mRNA (E, F) are observed in both germ layers. TR α mRNA (G, H) is exclusively expressed in the mesenchyme in contrast to TR β mRNA (I, J), which is expressed in the epithelium. All pictures were taken at the same magnification; scale bar = 500 μ m.

the endodermal part of the developing lung (d 15 of gestation) (Fig. 3, A and B). On d 18 of gestation, GR expression was ubiquitous in both epithelium and mesenchyme, albeit more pronounced in the epithelium (Fig. 4, A and B). The mRNA expression of RXR α and RXR β was detected in both mesenchyme and epithelium from d 15 of gestation onward (Fig. 3, C–F, and Fig. 4, C–F), which corroborates the immunohistochemical findings. In contrast to the broad expression of TR α in both epithelial and mesenchymal cells as shown by the immunohistochemical studies (Fig. 2, A–E), the expression of TR α mRNA in *in situ* hybridization studies was confined to the pulmonary mesenchyme (Fig. 3, G and H, and Fig. 4, G and H). The expression of TR β mRNA was detected predominantly in the pulmonary epithelium of the distal conducting airways (Fig. 3, I and J and Fig. 4, I and J). Expression of TR β mRNA was observed at all time points in pulmonary arteries, while expression of TR α mRNA was continuously observed in pulmonary veins. An accurate description of the expression patterns became more difficult near term (d 20 of gestation) because the walls of the airways become very thin due to

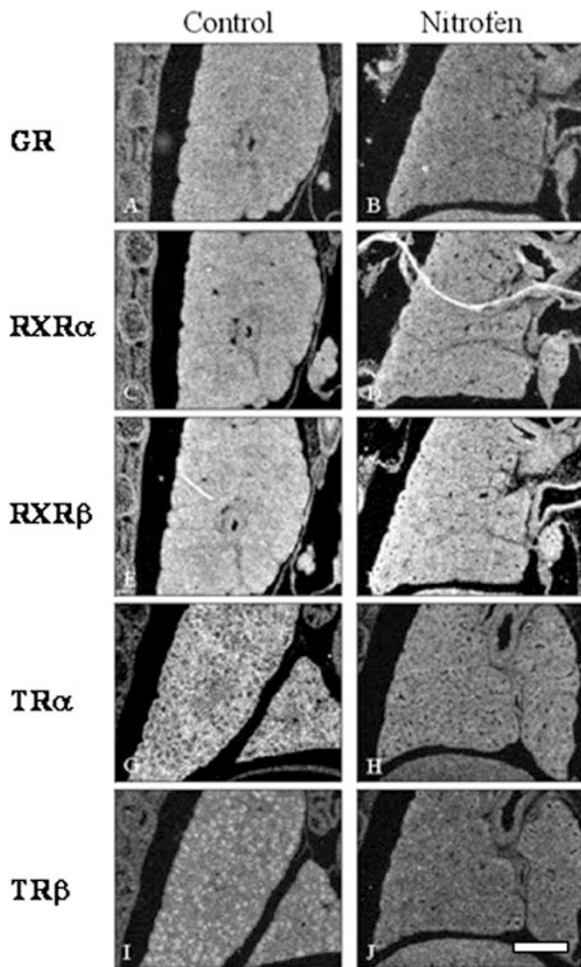


Figure 4. *In situ* hybridization studies of hormone receptor expression in normal (A, C, E, G) and nitrofen-induced CDH rat lungs (B, D, F, H) at d 18 of gestation. At this stage, GR mRNA is faintly expressed in the entire lung (A, B). RXR α mRNA (C, D) and RXR β mRNA (E, F) are expressed in both mesenchyme and epithelium. TR α mRNA (G, H) is expressed in the mesenchyme, whereas TR beta mRNA (I, J) is exclusively expressed in the epithelium. All pictures were taken at the same magnification; scale bar = 500 μ m.

expansion of the gas-exchange surface (results not shown). In nitrofen-induced rat lungs, the spatial and temporal expression pattern was similar to that observed in control lungs for all investigated receptors (Figs. 3 and 4).

DISCUSSION

In this present study, no obvious differences in the spatial and temporal expression pattern of GR α , TR, RAR (α, β, γ), and RXR (α, β, γ) were observed between human CDH lungs, pulmonary hypoplasia from other causes and control lungs. These results are supported by a series of experiments undertaken in the nitrofen rat model for CDH. This study was inspired by the promising results obtained by the administration of glucocorticoids, thyroid hormone or vitamin A to nitrofen-treated rats (13,16,17,25–27). In our previous study, we have shown the expression of the nuclear hormone receptors in human lung from 13.5 wk until term with no change in localization throughout development (21). However, there is

still limited information about the presence of these receptors in both human and rat CDH lungs.

Antenatal corticosteroids are recommended by National Institutes of Health consensus to all fetuses between 24 and 34 wk of gestation at risk of preterm delivery (10,18). This will lead to an induction of surfactant production, an acceleration of lung maturation and an increase in lung compliance. In the present study, we demonstrate that the GR α is indeed expressed in a similar fashion in both normal lung tissues as well as in hypoplastic lungs of CDH cases or pulmonary hypoplasia due to other causes. The same holds true for control rat lungs and nitrofen-induced CDH lungs. At mRNA level, using quantitative PCR, we found no significant difference in GR α expression between CDH and control lungs. These findings are suggestive for a potential similar responsiveness to GC in lungs of CDH fetuses. There was one report of three CDH patients that suggested the benefit of prenatal GC for fetuses with CDH (28). But recently, the randomized trial and cohort study by the CDH study group demonstrated no significant benefit of late prenatal GC (after 34 wk of gestation) to fetuses with CDH, although more cases have to be included (29). With the paucity of human data, the side effects of GC and the lack of information on dosage and timing, antenatal GC are not recommended beyond 34 wk of pregnancy (30,31).

The role of thyroid hormone in lung development is exerted predominantly during the later phases of lung development, in which the regulation of surfactant production takes place. In rats, administration of T₃ accelerates the process of septation, resulting in a greater alveolar surface area (32). Our study demonstrates that the localization of TR is not changed in hypoplastic lungs of fetuses with nitrofen-induced CDH. These results are compatible with the results from previous study by Tovar *et al.* (33), which demonstrated that nitrofen leads to decreased levels of T₃ and T₄ in the plasma but not at the tissue level. This is an indication that induction of pulmonary hypoplasia in nitrofen-induced CDH is not the result of an alteration in the localization of TR. In contrast to this, we observed lower mRNA expression of TR α 1 in human CDH lungs than in control lungs, which was in agreement with a previous study in nitrofen-induced CDH rats (34). Although the latter data are suggestive for a diminished TR α 1 expression, we are aware that far-reaching conclusions cannot be drawn due to the limitations of our study, in particular the limited number of CDH lung samples available for PCR analysis.

Vitamin A is important for lung development. *All-trans*-RA acts *via* RAR, whereas *9-cis*-RA exerts its effects *via* both RAR and RXR. RXR can act as homodimers or heterodimers with a variety of nuclear receptors, including RAR and TR (35,36). In animal studies, the lack or the excess of vitamin A during embryonic development results in congenital malformations such as infertility, anophthalmia, and lung hypoplasia (18,37,38). Transgenic RAR α /RXR α and RAR α / β double knockout mice were shown to have lung hypoplasia or agenesis (37–39). Our collaborative studies in the nitrofen rat model for CDH demonstrated that prenatal administration of vitamin A reduces the incidence of CDH and increases the levels of surfactant protein A and C in the offspring (16,17). In

human pneumocytes cultured experiments, vitamin A administered after nitrofen exposure significantly increased the expression of surfactant protein B (40). In our present study, we showed that the expression pattern for all RAR and RXR is not changed in hypoplastic lungs of nitrofen-induced CDH rats. Similar findings are shown in human tissues for all RAR and RXR by immunohistochemical studies. Owing to the limited number of CDH samples, no statistical analysis was done. Although we observed a low RXR γ mRNA expression in human CDH lungs, there is possibly a redundancy for most functions of RXR γ by the other receptors of the family. The studies in RAR/RXR mutant mice revealed that there is a high degree of functional redundancy among these receptors (36).

Our findings suggest that there is a potential for biologic effects of exogenous glucocorticoids, thyroid hormone, and retinoic acid in CDH lungs. Experimental studies have illustrated the potential benefits of antenatal corticosteroids and vitamin A to overcome pulmonary hypoplasia in animal models of CDH. Our results indicate that there might be a potential for similar effects in cases of human CDH patients as well. For instance, the current evaluation of vitamin A levels in newborn babies with CDH compared with age-matched controls (19) and the experimental data obtained by Greer *et al.* (41) potentially guide the use of prenatal modulation of pulmonary growth in human CDH in the near future. Very recently, a prospective international observational study on vitamin A levels in cord blood obtained institutional review board approval in Canada (Greer JJ, personal communication). Further advancement in therapies for CDH patients may arise from improved understanding of the mechanism of abnormal lung development (42).

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